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13. ABSTRACT (Maximum 200 Words) The retinoblastoma tumor suppressor protein (RB) participates in the growth regulation of breast cancer cells by controlling G ₁ -S phase progression and mediating cell cycle arrest in response to anti-mitogenic signaling. RB is functionally inactivated in nearly half of all breast cancers. Although RB loss has been implicated in the bypass of both DNA damaging and anti-estrogenic therapeutic pathways, studies of the impact of RB upon the therapeutic response of breast cancer have been limited. Thus, we believe that our analyses of RB function and its effect upon downstream targets in modifying the therapeutic response of breast cancer cells is imperative for the design of improved treatment strategies. We demonstrate using siRNA to <i>Rb</i> that its loss and subsequent target deregulation in MCF-7 cells contributes to resistance to anti-estrogen therapy and DNA damage checkpoint bypass following exposure to ionizing radiation (IR). Furthermore, microarray and proteomic analyses reveal that loss of cell cycle arrest following estrogen ablation in RB deficient cells is coupled with increased expression of downstream targets with respect to RB-proficient controls. Taken together, our data reveal that RB loss in breast cancer facilitates cellular resistance to two major modes of breast cancer therapy.				
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1. Introduction:

Breast cancer is the leading cancer diagnosis among American women impacting over 240,000 new patients per year. The retinoblastoma tumor suppressor protein, RB, is functionally inactivated in the majority of human cancers and nearly half of all breast cancers (1). RB participates in the growth regulation of breast cancer cells and it has been demonstrated that the inactivation of RB in mammary tumor models is associated with tumor progression. RB plays a central role in regulating the G₁/S phase progression (2). In quiescent cells, RB is hypophosphorylated and assembled in a transcriptional repressor complex to arrest cell cycle (3). In response to mitogenic factors, including estrogen in breast cancer, RB is inactivated through hyperphosphorylation catalyzed by the cyclin D-cdk4 and cyclinE-cdk2 complexes (4). These modifications are sufficient to disrupt interactions with E2F and initiate cell cycle progression (5,6). Moreover, both cyclin D1 and cyclin E are known to be overexpressed in 45% and 30% of breast cancers respectively (7,8), thereby increasing RB inactivation in breast carcinoma cells. In contrast, anti-mitogenic factors, such as DNA-damage and estrogen receptor (ER) antagonists, activate RB, inhibiting cell cycle progression. For example, RB activity is instrumental in the DNA-damage induced cell cycle checkpoint and is necessary for the induction of G₁ and S-phase arrest following DNA damaging events (9,10). It is believed that RB elicits this checkpoint by modulating the activity of transcription factors such as members of the E2F protein family (9). Therefore, in breast cancer, loss of RB function could lead to downstream target gene deregulation, a DNA-damage checkpoint deficiency, and bypass of the ER-ablation pathway.

While RB has been shown to be important in carcinogenesis and is a modifier in the therapeutic response, exploration of the function of RB in breast cancer therapy has been limited. In an attempt to elucidate the activity of RB in the therapeutic response of breast cancer cells, it is important to understand how estrogen-dependent and independent tumors are most commonly treated. So as to limit the amount of mitogenic hormone to which the tumor is exposed, estrogen-dependent tumors are treated with chemical antiestrogens, primarily tamoxifen. However, RB loss has been thought to participate in disruption of this pathway and resistance to treatment. Because tamoxifen resistance is characteristic of 50% of estrogen receptor (ER)-positive breast cancers (11), it remains a significant clinical problem while the mechanism through which it occurs remains elusive. Thus, examining the function of RB in response to estrogen ablation has strong clinical relevance. Estrogen-independent tumors have traditionally been treated using DNA-damaging agents, such as chemotherapeutics and ionizing radiation (12). However, RB-deficient cells have an impaired response to these DNA-damaging agents used in the treatment of breast cancer. Our lab has previously demonstrated in primary cells that RB down-regulates specific target genes and elicits cell cycle inhibition in response to DNA-damaging agents (13, 14). Therefore, we feel that studying the importance of RB in the response of estrogen-independent tumors to these traditional therapies will provide new insight into current breast cancer therapy.

2. Body:

Aim 1: Recapitulate RB loss in estrogen-dependent breast cancer cells. To recapitulate RB loss in the MCF-7 and T-47D breast cancer model systems, siRNA molecules containing a short hairpin sequence complementary to *Rb* (bp 1261-1279, in the A/B pocket of the protein) or a control lacking the short hairpin sequence (obtained from Dr. Scott Lowe, Cold Spring Harbor Lab) were transfected into cells. Puromycin resistant stable clones were selected and validated for *Rb* gene silencing. RB down-regulation was assessed in MCF-7 clones using immunoblot and immunofluorescence for RB (Fig. 1A). Screening for T-47D RB-deficient colonies is still underway due to poor transfection efficiency and cell death associated with selection. However, 14 T-47D clones of each type are currently being cultured and screened.

Subsequently, the consequences of RB loss on target gene expression in the MCF-7 clones were analyzed by immunoblot and immunofluorescence. Our data demonstrate deregulated expression of many target genes such as, cyclin A, cyclin E, PCNA, MCM-7, and cyclin B1 in the absence of RB (Fig 1B).

Aim 2: Assess the action of RB in the therapeutic response of breast cancer cells to estrogen antagonists and ionizing radiation. The RB-proficient and -deficient MCF-7 clones were used to investigate the impact of RB loss and target deregulation on the cell cycle response to estrogen blockade and DNA-damage *in vitro*. To analyze cell cycle progression, cells were labeled with bromodeoxyuridine (BrdU) for 12 h and incorporation was detected using immunofluorescence. BrdU incorporation revealed that RB loss allows for abrogation of cell cycle arrest in response to estrogen depletion via culturing cells in charcoal dextran treated (CDT) (10%) media and/or tamoxifen (10^{-6} M) treatment. Furthermore, our data indicate that RB loss and subsequent target deregulation contribute to DNA damage checkpoint bypass 18 h following exposure to 5 Gy ionizing radiation (Fig. 2A). The cell cycle profiles of these cells were analyzed following exposure to control media containing FBS, or estrogen depleted media containing CDT/ tamoxifen, or 2 hr post 5 Gy ionizing radiation by flow cytometry. The results confirm that RB loss abrogates both DNA damage and hormone ablation checkpoint function as RB deficient cells were able to undergo S-phase post treatment similar to untreated controls (Fig. 2B). Loss of checkpoint in RB-deficient cells is coupled with heightened E2F activity following hormone deprivation as determined by activation of a transfected 3X E2F-luciferase reporter construct (Fig. 2C).

In addition to the approved statement of work, genomic and proteomic analyses with these clones are underway to reveal the mechanisms by which RB loss influences therapeutic resistance. The Introduction to Functional Genomics Course offered at the University of Cincinnati has funded our efforts to investigate the gene expression profile differences between control MCF-7 cells and their siRB counterparts following culture in control media, CDT/ tamoxifen, and 18 h following 5 Gy ionizing radiation using Affymetrix microarray chips. Data analysis is currently underway, however, the initial results reveal efficient downregulation of RB in the siRB cells and significant differences in the profile trends between the RB proficient and deficient cells following hormone ablation and DNA damage (Fig 3). In parallel, proteomic experiments are underway to analyze any

differences in the protein expression patterns in the MCF-7 control cells, and siRB cells. Two dimensional gel electrophoresis has been performed on these samples in triplicate and protein spots of interest are being picked and digested for identification by mass spectrometry by the University of Cincinnati Proteomics core. Protein identification is currently underway, however, at first glance, general protein expression tends to increase from the MCF-7 donor control to the siRB knockout. Additionally, there are dozens of proteins that are only present or significantly upregulated in the siRB MCF-7 cells (Fig 4).

Aim 3: Determine the response of breast cancer cell lines with varied RB status xenografted into ovariectomized athymic nude mice to anti-estrogens and ionizing radiation. Currently, xenografts in ovariectomized nude mice are being employed to elucidate the significance of RB loss on therapeutic responsiveness *in vivo*. The initial cell implants have occurred, however, no data has been collected regarding tumor growth.

3. Key Accomplishments:

| Task 1. Recapitulate RB loss in breast cancer model systems.

a. Transfected siRNA molecule into MCF-7 and T-47D cells to inactivate RB in stable clones.

b. Validation of RB loss by immunoblot and immunofluorescence is complete in MCF-7 clones and is underway in T-47D clones.

c. Deregulation of RB targets was evident by immunoblot in MCF-7 siRNA clones.

| Task 2. Elucidate the action of RB in the therapeutic response of estrogen-dependent tumors.

a. RB loss and subsequent target deregulation disrupts estrogen-dependent proliferation pathways, promoting growth in the absence of estrogen or the presence of tamoxifen.

b. RB loss abrogates the DNA damage checkpoint that is evident in control MCF-7 clones.

| Task 3. Determine the response of breast cancer cell lines with varied RB status xenografted into ovariectomized athymic nude mice to anti-estrogens and ionizing radiation.

a. Athymic ovariectomized nude mice have been obtained and initial experiments are underway to analyze the tumorigenicity of the MCF-7 clones.

4. Reportable Outcomes:

AWARD:

Albert J. Ryan Foundation Fellowship (2004-2006)

PUBLICATIONS:

Bosco, EE, Knudsen ES. (2005) Differential Role of RB in Response to UV and IR Damage. *Nuc Acids Res.* In Press.

Mayhew CN, Bosco EE, Fox SR, Okaya T, Tarapore P, Schwemberger SJ, Babcock GF, Lentsch AB, Fukasawa K, Knudsen ES. (2005) Liver-specific pRB Loss Results in Ectopic Cell Cycle Entry and Aberrant Ploidy. *Cancer Research.* In Press

ABSTRACTS/ POSTER PRESENTATIONS:

University of Cincinnati, College of Medicine Poster Forum (Nov 2004)

University of Cincinnati, Cell Biology Graduate Retreat (Nov 2004)

ASM DNA Repair and Mutagenesis Conference, Southampton Bermuda (Nov 2004)

5. Conclusions:

Taken together, our data reveal that loss of RB function in breast cancer facilitates cellular resistance to both hormone ablation and DNA damage therapies. Therefore, these studies uncover a possible mechanism through which breast cancer cells develop therapeutic resistance. Our goal for these findings, in conjunction with the data from genomics, proteomics, and the mouse model, is to allow for the design of more efficacious therapeutics which improve patient prognoses.

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7. Appendices:

Figure 1

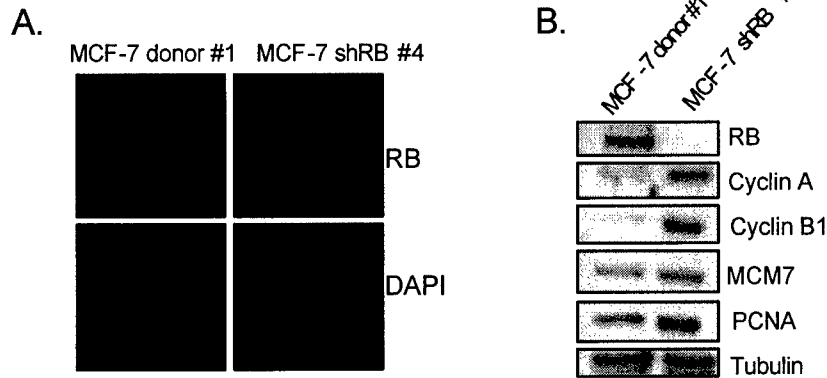


Fig 1. MCF-7 clones containing shRB molecule yield stable RB downregulation. (A) Immunofluorescence using an anti-RB antibody was performed on stable MCF-7 cells containing the donor control vector or shRB vector. (B) Asynchronously proliferating MCF-7 donor or shRB clones were harvested in RIPA buffer. Protein concentrations were normalized using BioRad DC Assay. Equal amounts of protein were separated by electrophoresis and immunoblotting for RB, cyclin A, cyclin B1, MCM7, PCNA was performed. Lysates were immunoblotted for tubulin to confirm equal loading.

Figure 2

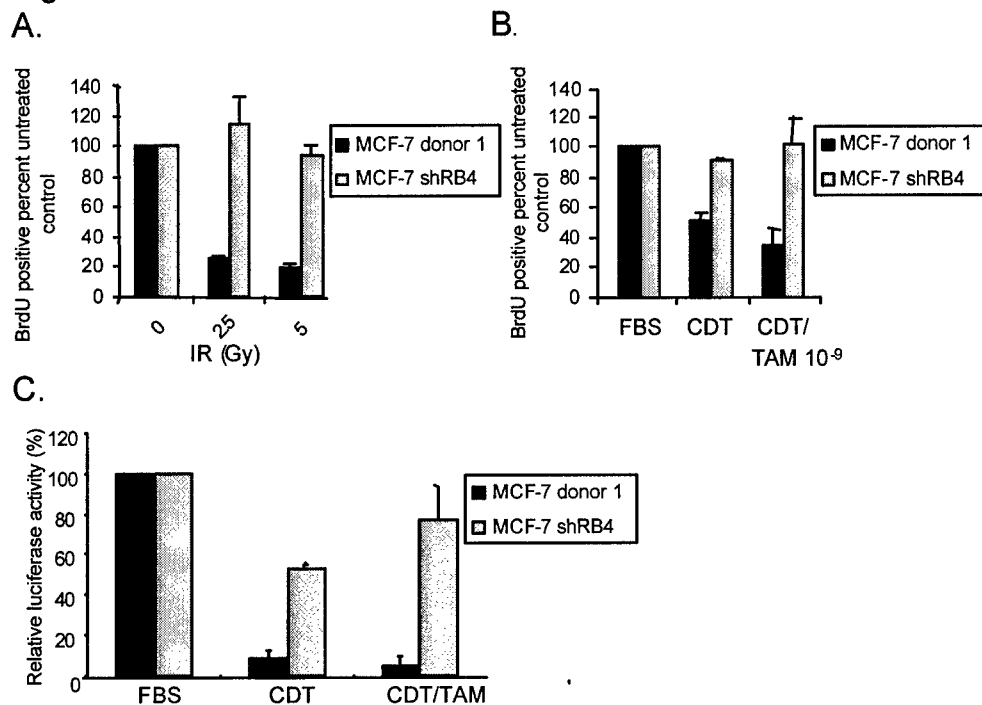


Fig 2. RB downregulation contributes to anti-mitogenic checkpoint abrogation in MCF-7 cells. (A) Stable MCF-7 cells containing the donor control vector or shRB vector were exposed to 0, 2.5, or 5 Gy ionizing radiation and cultured for 18h, in the presence of BrdU for the final 8h. The proliferative fraction of treated cells with respect to untreated controls was determined through immunofluorescence. (B) Stable donor or shRB MCF-7 cells were cultured in medium containing control FBS, charcoal dextran treated serum (CDT), or CDT+ tamoxifen 10^{-9} for 3 days. Cells were pulsed with BrdU and processed as in (A). (C) Stable donor or shRB MCF-7 cells were transfected with a 3xRE2F-luciferase reporter and β -galactosidase. Post-transfection cells were cultured in FBS, CDT, or CDT+ tamoxifen 10^{-9} for 3 days. Cells were harvested and analyzed for β -galactosidase and luciferase activity. Activity of cells in FBS was set to 100%, and served as a normalization factor for other samples.

Figure 3

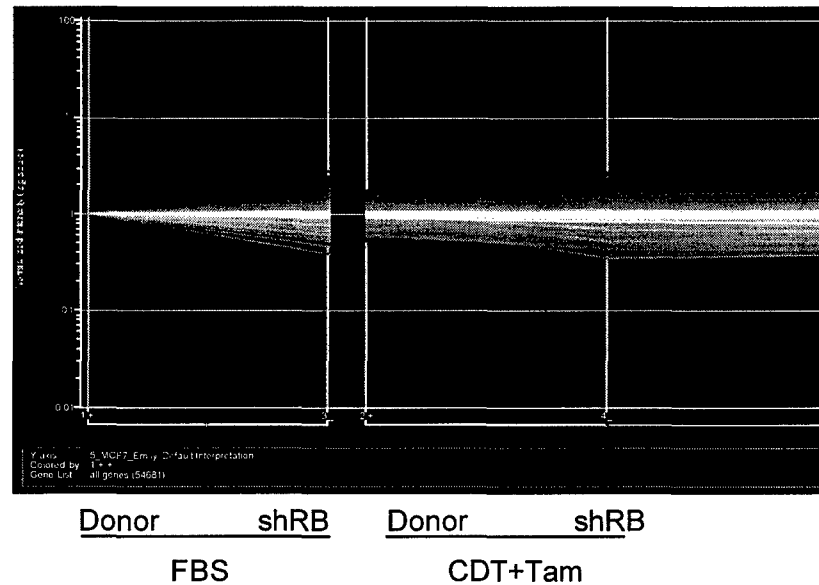


Fig 3. RB loss influences genetic deregulation. Affymetrix array chip of MCF-7 donor or shRB cells cultured in control FBS or CDT+ Tamoxifen 10^{-9} for 3 days. All samples are normalized to donor cells in FBS (set to 1).

Figure 4

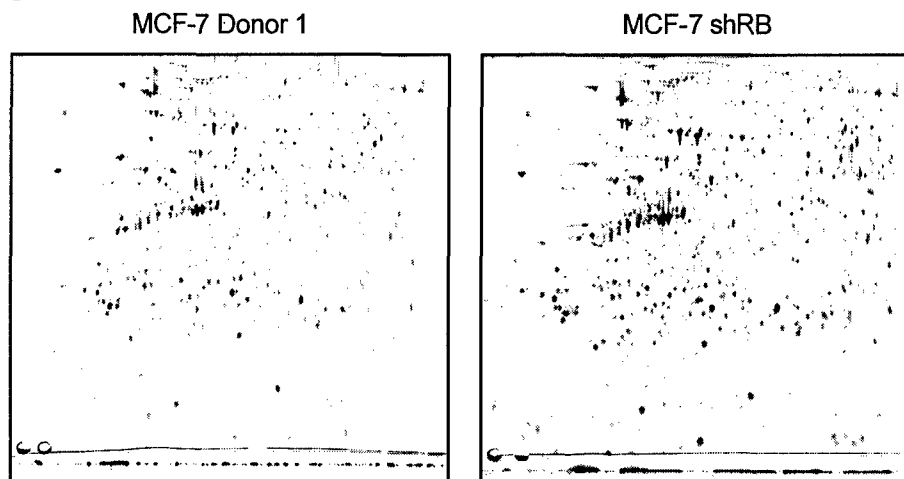


Fig 4. RB loss influences protein expression patterns. MCF-7 donor and shRB cells cultured in media containing control FBS were harvested and lysed. Equal protein concentrations were loaded onto gels in triplicate for 2-dimensional gel electrophoresis. The gels were silver stained and averaged to determine the relative spot intensities. Protein spots of interest are being picked for trypsinization and identification using MALDI mass spectrophotometry.